ORIGINAL ARTICLE

Antioxidant activity and protective effect against oxidative hemolysis of *Clinacanthus nutans* (Burm.f) Lindau.

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Abstract

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Increasing evidence suggests that oxidative damage to cell components has an important pathophysiological role in many human diseases. The free radicals formed in cells can readily attack protein, DNA and unsaturated lipids resulting in their loss of function and damage. Red blood cells are highly susceptible to oxidative damage which results in cell lysis. A natural antioxidant could be a potential therapeutic intervention. Thus, we examined the antioxidant activity of *Clinacanthus nutans* (CN). An ethanolic extract of dried leaves of CN was used in this study. The free radical (1,1-diphenyl-2-picrylhydrazyl; DPPH) scavenging activity, the ferric reducing antioxidant power (FRAP) and the intracellularly antioxidant activity of the

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extract were determined. The protective effect of the extract against 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH)-induced rat red blood cell lysis was also evaluated. It was found that the extract could scavenge DPPH with the maximum scavenging activity of 67.65±6.59% and with an IC_{50} of 110.4±6.59 μg/ml. The FRAP value was 17 mg ascorbate equivalent to one gram of the extract. The extract demonstrated a significant inhibition of peroxide production in rat macrophages stimulated by phorbol myristate acetate (PMA) and protected red blood cell against AAPH-induced hemolysis with an IC_{50} of 359.38±14.02 mg/ml. In conclusion, the ethanolic extract of CN had an antioxidant activity and protective effect against free-radical-induced hemolysis.

Key words : free radicals, antioxidants, *Clinacanthus nutans*, hemolysis

บทคัดย่อ

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ฤทธิ์ต้านออกซิเดชันและป้องกันเม็ดเลือดแดงแตกเนื่องจากออกซิเดชันของพญายอ

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มีหลักฐานยืนยันว่าภาวะที่มีการสร้างอนุมูลอิสระมากเกินเป็นกลไกสำคัญกลไกหนึ่งที่ก่อให้เกิดพยาธิสภาพของโรคต่าง ๆ อนุมูลอิสระที่สร้างขึ้นภายในเซลล์จะทำปฏิกิริยากับโปรตีน ดีเอ็นเอ และกรดไขมันชนิดที่ไม่อิ่มตัว ทำให้เซลล์นั้น ๆ เสียหน้าที่และเป็นอันตรายต่อเซลล์ได้ เม็ดเลือดแดงเป็นเซลล์ที่ไวต่อการทำลายโดยอนุมูลอิสระจึงอาจแตกได้ง่ายเมื่อมีอนุมูลอิสระมากเกิน การใช้สารต้านออกซิแดนท์จากธรรมชาติอาจจะเป็นวิธีการที่ดีในการป้องกันรักษาเซลล์บาดเจ็บเนื่องจากอนุมูลอิสระ คณะผู้วิจัยได้ตรวจสอบหาฤทธิ์ต้านอนุมูลอิสระของพญายอ (*Clinacanthus nutans*; CN) สารสกัดของใบแห้งพญายอด้วยแอลกอฮอล์นำมาใช้ในการศึกษาครั้งนี้ ตรวจสอบความสามารถของสารสกัดในการเก็บจับ (scavenging) อนุมูลอิสระ ซึ่งพบว่าสารสกัดสามารถเก็บจับอนุมูลอิสระ 1,1-diphenyl-2-picryl-hydrazyl (DPPH) ได้โดยมีฤทธิ์เก็บจับสูงสุดคิดเป็นร้อยละ 67.65±6.59 และมีค่า IC_{50} เท่ากับ 110.40±6.59 มคก./มล. ตรวจสอบความสามารถในการรีดิวส์ โดยวิธี ferric reducing antioxidant power (FRAP) ซึ่งได้ว่าสารสกัด 1 กรัม มีความสามารถในการรีดิวส์เทียบเท่ากับแอสคอเบท 17 มก. และตรวจสอบฤทธิ์ต่อการสร้าง peroxides ภายในเซลล์ macrophages ของหนูขาวเมื่อกระตุ้นด้วยสาร phorbol myristate acetate (PMA) ซึ่งพบว่าสารสกัดสามารถยับยั้งการสร้าง peroxides ได้อย่างมีนัยสำคัญทางสถิติ และทดสอบความสามารถของสารสกัดในการป้องกันเม็ดเลือดแดงแตกเนื่องจากอนุมูลอิสระสร้างจากสาร AAPH ซึ่งพบว่าสารสกัดสามารถป้องกันได้เป็นอย่างดี โดยมีค่า IC_{50} เท่ากับ 359.38±14.02 มคก./มล. ผลการทดลองสรุปได้ว่าสารสกัดจากใบพญายอมีฤทธิ์ต้านออกซิแดนท์และสามารถป้องกันการแตกของเซลล์เม็ดเลือดแดงอันเนื่องจากอนุมูลอิสระได้

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Reactive oxygen species (ROS), such as superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$) and peroxyl radical ($ROO^{\bullet}$), are constantly generated *in vivo* both by endogenous aerobic metabolism and exposure to exogenous sources such as UV irradiation, environmental pollutants and diets. However, aerobic organisms have many substances, such as superoxide dismutase, catalase, vitamin C and vitamin E, that delay or inhibit oxidative damage to a target molecule namely antioxidant (Halliwell & Gutteridge, 1990). The imbalance of oxidants and antioxidants of the

body leads to an oxidative stress resulting in destruction of unsaturated lipids, DNA, proteins and other essential molecules. Increasing evidence suggests that oxidative damage to cell components has a relevant pathophysiological role in several types of human diseases (Ames *et al*., 1993). Free radicals have been reported to cause red blood cell lysis in patients with blood pathologies such as thalassemia (Vives-Corrons *et al*., 1995). The erythrocytes are highly susceptible to oxidative damage due to the high polyunsaturated fatty acid content of their membrane and the high cellular concentration of oxygen and hemoglobin, all of which are powerful promoters of oxidative processes (Clemens *et al*., 1987). Exposure of erythrocytes to free radicals leads to a number of membrane changes including lipid peroxidation (Koster & Slee, 1983; Lal *et al*., 1980), reduction in deformability (Kurata *et al*., 1994), changes in cell morphology (Shinar *et al*., 1989), protein cross-linking and fragmentation (Vissers *et al*., 1994). These are the most common configuration damage leading to lysis of red blood cells.

Focussing our attention on natural sources of antioxidants for the protection of the body from oxidative stress, we investigated the antioxidant activity and the protective effect of the ethanolic extract of *Clinacanthus nutans* (Burm. F.) Lindau. against free radical-induced hemolysis. The *Clinacanthus nutans* (CN) is a well-known medicinal plant in Thai folklore medicine (Thai name: phaya yaw) for the treatment of a variety of symptoms such as herpes infectious, (Sangkitporn *et al*., 1995; Janwitayanuchit *et al*., 2003), inflammation, skin pruritis and insect bites (Janwitayanuchit *et al*., 2003). In this study we investigated the antioxidant activity of CN extract by examining its free radical scavenging activity, ferric reducing power and inhibitory effect on PMA-induced ROS production in rat macrophages. We also treated rat red blood cells with AAPH which generated free radicals at a constant rate in a water environment by unimolecular thermal decomposition without the addition of potentially interfering cofactors and transition metals (Landi *et al*., 1995). Then we tested the protective effect of the CN extract against AAPH-induced hemolysis.

Materials and methods

1. Plant extraction

Leaves of CN were collected from Amphur Pakchong, Nakorn Rajchasema, a province in the northeast of Thailand. Dried leaves were pulverized and then soaked in 50% ethanol for 7 days. The alcoholic solvent was evaporated under low pressure at 60ºC, after that the solution was dried by lyophilization. The water-soluble product obtained was a greenish powder with the percent yield of 11.06.

A plant specimen was identified by Mr Charan Leeratiwong, Department of Biology, Faculty of Science, Prince of Songkla University, and the voucher specimen No. SKP 0010314 was deposited at the Herbarium of Pharmacognosy and Pharmaceutical Botany Department, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkla, Thailand.

2. Measurement of antioxidant activity

2.1 Free radical scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical which has maximum optical absorbance at 515 nm. The reaction of DPPH with free radicals scavenger causes a decline in the absorbance value (Mathiesen *et al*., 1995). Ethanolic extract of CN at various concentrations (1-300 µg/ml; final concentration) was added to equal volume of 0.1 mM DPPH solution in ethanol (0.05 mM; final concentration) and left for 20 min at room temperature. The absorbance of the mixture was measured at 515 nm. Blanks contained ethanol and CN extract at corresponding concentrations. Ascorbic acid was used as a positive control. These measurements were run in duplicate. The % scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = [(A_{DPPH}-A_{TEST})/A_{DPPH}] \times 100$$

Where A_{DPPH} is the absorbance of DPPH without CN extract (control), and A_{TEST} is the absorbance of DPPH in the presence of CN extract.

2.2 Ferric reducing antioxidant power assay (FRAP assay)

FRAP assay was carried out by the method of Benzie & Strain (1996) with slight modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to its ferrous (Fe^{2+}-TPTZ), intensive blue colored form in the presence of antioxidant. Three hundred millimolar acetate buffer, pH 3.6, 10 mM TPTZ and 20 mM $Fe.Cl_3.6H_2O$ were mixed in a ratio of 10:1:1 to be a working FRAP reagent. Ten microliters of CN extract (1-100 µg/ml) were mixed with 300 µl of FRAP reagent and incubated at 30ºC for 4 min. The absorbance at 610 nm was monitored by Automate Model RA 100 (Technicon Instruments Corporation, USA). All reagents were freshly prepared before used. The standard curve for FRAP assay was generated by using ascorbic acid as reducing agent.

2.3 Effect of CN extract on phorbol myristate acetate (PMA)-induced reactive oxygen species (ROS) production in rat macrophages

2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) is a fluorogenic probe used to detect intracellular ROS. This method depends on the deacetylation of H_2DCF-DA by intracellular esterases to form the oxidant sensitive compound, 2,7-dichlorodihydrofluorescein (H_2DCF). Then H_2DCF was oxidized by H_2O_2 to be 2,7-dichlorofluorescein (DCF), a fluorescent product (Brubacher & Bols, 2001).

Male Sprague-Dawley rats weighing 250-300 g were obtained from the Experimental Animal Unit, Faculty of Medicine, Khon Kaen University, Thailand. The animals were fed with standard rat chow (CP company, Thailand) and housed in an enviroment of 25-28ºC with a 12-h light: 12-h dark cycle starting at 06.00 am. All animal experiments were conducted in accordance with the general guideline for the care and use of laboratory animals of Khon Kaen University Animal Ethic Committee. The experimental protocol has been approved by Animal Ethic Committee of Khon Kaen University.

The rats were anesthetized with pentobarbital sodium (60 mg/kgBW, i.p.). The macrophages were collected from intraperitoneal cavity and suspended in Hank's Balanced Salt Solution (HBSS) and adjusted to $2x10^6$ cells/ml. Cell viability was examined by using trypan blue exclusion test. Only the cell suspension with at least 80% viability was used in the experiment. Catalase (100 U/ml, final concentration) was added to 400 µl of cell suspension and incubated at 37ºC for 10 min. After adding CN extract at various concentrations (10, 30, 100 and 300 µg/ml) and the fluorescence probe H_2DCF-DA (4.78 µM final concentration), the mixture was further incubated for 15 min. Then the mixture was incubated with the free radical stimulating agent, phorbol myristate acetate (PMA, 0.65 µM final concentration) for 45 min. The fluorescence intensity of DCF was measured by spectrofluorometer at the excitation and emission wavelengths of 485 and 514 nm, respectively (Vowells *et al*., 1995). The fluorescence intensity was proportional to the amount of free radicals. For the positive and negative control, 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron, 300 µg/ml) and HBSS were used instead of CN extract respectively.

3. Investigation of the protective effect of CN extract against free radical induced red blood cell lysis.

3.1 Preparation of red blood cells

Rats were anesthetized with pentobarbital sodium (60 mg/kgBW, i.p.) and the red blood cells (RBC) were collected from the abdominal aorta. The RBC were separated from plasma by centrifugation at 1500 g for 10 min and washed three times with five volumes of phosphate buffer saline (PBS, pH 7.4) and diluted to be 20% cell suspension.

3.2 Lysis of red blood cells

Oxidative hemolysis was induced by a peroxyl radicals initiator, AAPH (He *et al*., 2000). AAPH caused an oxidation of lipids and proteins in cell membrane resulting in lysis of RBC. Five hundred microliters of RBC suspension was mixed with 500 µl of CN extract at various concentrations

(200-1000 µg/ml), then 250 µl of 400 mM AAPH was added. The mixture was incubated at 37ºC for 3 hr in a water bath. After incubation, 2 ml of PBS was added into the reaction mixture followed by centrifugation at 2000 g for 10 min. The absorbance of the supernatant at 540 nm was measured by using spectrophotometer. Percentage of inhibition was calculated by the following equation.

$$\% \text{ Inhibition} = [\{A_{ctl}-A_{test}\}/A_{ctl}] \times 100$$

A_{ctl} is the absorbance of the sample without CN extract and A_{test} is the absorbance of the sample with the CN extract. For the positive control, ascorbic acid was used instead of the CN extract.

4. Chemicals

Ascorbic acid (vitamin C), bovine liver catalase, ferric chloride hexahydrate, ferrous chloride tetrahydrate and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co., (St. Louis, MO, USA). 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Aldrich Chemical Company Inc., (Milwaukee, WI, USA). Dichlorodihydrofluorescein diacetate (H_2DCF-DA), 4,5-dihydroxy-1,3-benzenedisulfonic acid (Tiron) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Fluka Chemika (AG, Switzerland). HBSS composed of 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.25 mM Na_2HPO_4, 1.0 mM $MgSO_4$, 1.3 mM $CaCl_2$, 4.2 mM $NaCHO_3$ and 5.6 mM Glucose.

5. Statistical analysis

The data were presented as mean ± S.E.M. of at least four experiments and analyzed by one-way ANOVA followed by Newman Keuls test to determine significant difference between groups at $p<0.05$. IC_{50} was calculated by nonlinear regression fitting into Hill's equation using the program SigmaPlot (version 8, SPSS Science Inc.).

Results

1. Free radical scavenging activity of CN extract

The CN extract and ascorbic acid scavenged DPPH in a dose-dependent manner with an IC_{50} of 110.40±6.59 and 9.72±0.56 µg/ml respectively (Figure 1). The CN extract showed a moderate scavenging activity with the maximum effect of 67.65±6.59% and the potency was approximately 0.08 times of ascorbic acid.

2. Ferric reducing antioxidant power (FRAP) activity of CN extract

CN extract possessed reducing power, with 1 g of CN extract being equivalent to 17 mg of ascorbic acid. The ferric reducing activity of CN

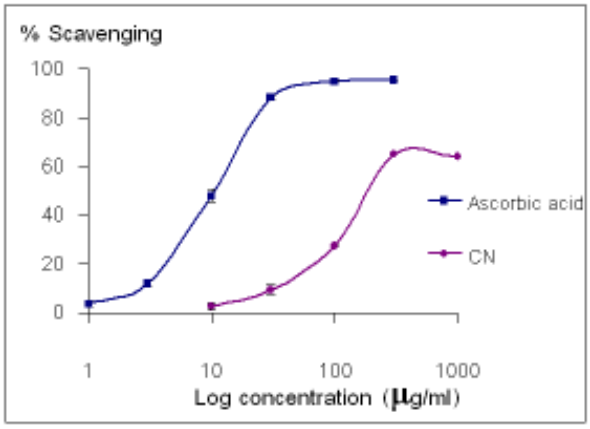


Figure 1. The free radical scavenging activity of the ethanolic extract of CN and ascorbic acid examining by DPPH method. Results are mean ± S.E.M of six experiments. (CN: *Clinacanthus nutans*)

extract was 59 times less potent than ascorbic acid. The plot for reducing Fe^{3+}-TPTZ reagent by the CN extract is shown in Figure 2.

3. Inhibitory effect of CN extract on PMA-induced free radical production in rat macrophages.

In order to examine the toxicity of CN extract to macrophages, the CN extract at concentrations of 10, 30, 100 and 300 µg/ml were added to cell suspension and incubated for 70 min. The results showed no significant difference in percent cell viability among control and CN extract treated groups. The fluorescent intensity of PMA treated group was significantly higher than that of control group (78.15±15.94 and 34.25±6.94 respectively,

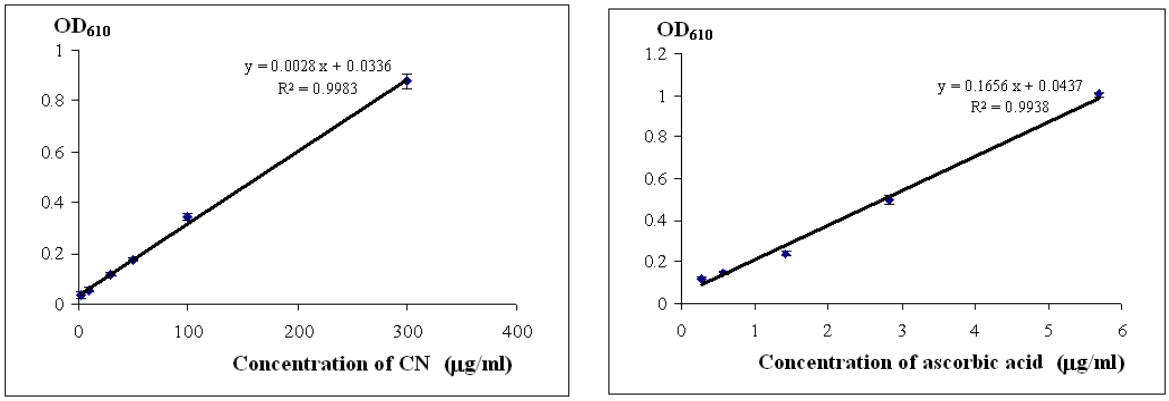


Figure 2. The ferric-reducing antioxidant power of the ethanolic extract of CN (top) and ascorbic acid (bottom). The CN extract and ascorbic acid reduced ferric to ferrous which has the optical absorbance at 610 nm, therefore, the increasing in absorbance indicated the ferric reducing activity.
(CN: *Clinacanthus nutans*)

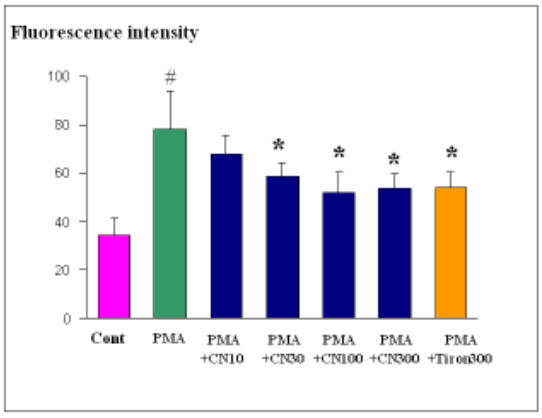


Figure 3. Effects of the ethanolic extract of CN and tiron on the free radical production in PMA-stimulated macrophages. CN at concentrations of 30, 100 and 300 µg/ml and also tiron 300 µg/ml significantly inhibited the PMA-stimulated free radical production.
(CN: *Clinacanthus nutans*, Cont: control group, PMA: phorbol-myristate acetate 0.65 µM, #: $P<0.05$ as compared to control by ANOVA, *: $P<0.05$ as compared to the group adding PMA alone by ANOVA)

Figure 3), indicating that PMA 0.65 µM could significantly stimulate the macrophages to produce free radicals. Interestingly, the CN extract at concentrations of 30, 100 and 300 µg/ml significantly reduced PMA-induced free radical production with the fluorescent intensity of 58.72±5.52, 51.92± 8.49 and 53.50±6.17 respectively (Figure 3). Tiron, a specific scavenger for superoxide anion ($O_2^{\bullet -}$) also exerted significant reduction of PMA-induced free radical production with the fluorescent intensity of 54.10±6.37.

4. Effect of CN extract on AAPH induced-hemolysis.

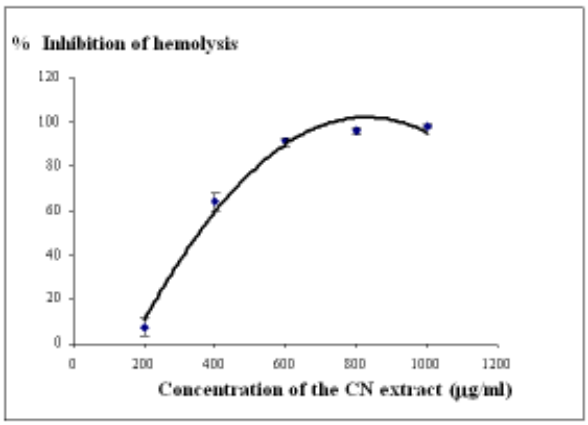
After 3 hours of incubation with AAPH, approximately 95% of erythrocytes were lysed. The protective effects of CN extract and ascorbic acid on the hemolysis induced by AAPH are shown in Figure 4 presenting the percentage of hemolysis inhibition at various concentrations. IC_{50} of the CN extract and ascorbic acid were 359.38±14.02 and 13.37±0.57 µg/ml respectively. The CN extract had a maximum inhibitory effect of 98.07 ±0.91%.

Discussion

This study has demonstrated the antioxidant activity and the protective effect against oxidative-induced hemolysis of the CN extract. The CN extract had free radicals scavenging activity, ferric reducing antioxidant power and intracellular inhibitory effect on PMA-induced free radical production.

Polyphenols, tannins and flavonoids are very valuable plant constituents possessing scavenging action due to their several phenolic hydroxyl groups (Hatano *et al*., 1989). Although C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin 7-*O*-β-glucopyranoside, orientin, isoorientin and sulfur containing glucosides have been isolated from the butanol and water-soluble portion of the methanol extract of the stem and leaves of CN collected in Thailand (Teshima *et al*., 1998), the constituents of CN extract which possess the free radical scavenging activity have not been well established. The CN extract also has reductive capability on reducing Fe^{3+} to Fe^{2+} in the FRAP assay showing its ability in donating an electron.

The reductions of oxidation of fluorescence probe or of fluorescent intensity induced by PMA in macrophage cells in the presence of CN extract could be due to (i) an inhibitory effect on free radical production, (ii) a free radical scavenging activity and (iii) a cytotoxic effect of the CN extract. The third probability is unlikely because CN extract did not show any toxicity on cell suspension. In this experiment, PMA was used to stimulate the



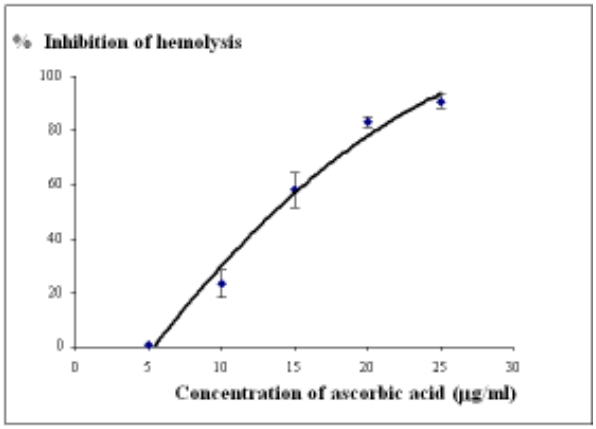


Figure 4. Effects of the ethanolic extract of CN and ascorbic acid on AAPH-induced hemolysis.
(CN: *Clinacanthus nutans*)

production of ROS and H_2O_2 in macrophages. PMA directly activates protein kinase C resulting in phosphorylation of several proteins which in turn activates NADPH oxidase. The NADPH oxidase then catalyzes the one-electron reduction of oxygen to $O_2^{\bullet -}$ (Fabiani *et al*., 1998). Therefore, it is possible that the CN extract may affect one of these processes.

Although the inhibitory effect of the CN extract on free radical production promoted by AAPH and PMA is possible, but the free radical scavenging effect of the extract is more likely. This is supported by the results that this extract effectively scavenged DPPH, a stable free radical and it also exhibited significant FRAP activity. If the mechanism of the antioxidant action of the extract was to inhibit free radical production, it may not be able to counteract the oxidizing effect of direct oxidant such as DPPH or ferric species as shown in this study. Therefore, the plausible antioxidant effect of the CN extract may be due to its free radical scavenging activity.

Oxidative damage of erythrocytes membrane (lipid and protein peroxidation) may be implicated in hemolysis associated with some hemoglobinopathies, oxidative drugs, transition metal excess, radiation and deficiencies in some erythrocyte antioxidant systems (Ko *et al*., 1997). In this study, we found that the ethanolic extract of CN could inhibit the AAPH-induced red blood cell lysis. AAPH, a water soluble free radical generator, was used to imitate the in vivo condition of oxidative stress. The peroxyl radicals are generated by thermal decomposition of an azo compound in oxygen and cause lipid peroxidation of red blood cell membrane resulting in cell lysis. In addition oxidant may decrease erythrocyte deformations leading to decrease survival of erythrocyte and circulatory impairments (Halliwell & Gutteridge, 1990). The protective effect of the CN extract on red blood cell lysis induced by AAPH may be owing to the antioxidant activity of this extract.

In conclusion, the ethanolic extract of CN has *in vitro* antioxidant activity and reduced AAPH-induced red blood cell lysis. These results indicate the possibility of employing the CN extract as an antioxidant substance to ameliorate the oxidative damage. However, further investigations of its *in vivo* activity are necessary.

Acknowledgement

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